PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C08B 37/00, A61K 47/48 | A1

(11) International Publication Number: WO 98/05689

(43) International Publication Date: 12 February 1998 (12.02.98)

(21) International Application Number: PCT/US97/13475

(22) International Filing Date: 28 July 1997 (28.07.97)

(30) Priority Data:
08/691,862 2 August 1996 (02.08.96) US

(71) Applicant: DUKE UNIVERSITY MEDICAL CENTER [US/US]; Erwin Road, Durham, NC 27710 (US).

(72) Inventors: STAMLER, Jonathan, S.; 3416 Juniper Place, Chapel Hill, NC 27514 (US). TOONE, Eric, J.; 2601 Evans Street, Durham, NC 27705 (US). STACK, Richard, S.; 106 Alder Place, Chapel Hill, NC 27514 (US).

(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: AU, CA, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: POLYMERS FOR DELIVERING NITRIC OXIDE IN VIVO

(57) Abstract

Disclosed are novel polymers derivatized with at least one -NOx group per 1200 atomic mass unit of the polymer. X is one or two. In one embodiment, the polymer is an *S*-nitrosylated polymer and is prepared by reacting a polythiolated polymer with a nitrosylating agent under conditions suitable for nitrosylating free thiol groups. The polymers of the present invention can be used to coat medical devices to deliver nitric oxide *in vivo* to treatment sites.

POLYMERS FOR DELIVERING NITRIC OXIDE IN VIVO

Background of the Invention

Many modern medical procedures require that synthetic medical devices remain in an individual undergoing treatment. For example, coronary and peripheral procedures involve the insertion of diagnostic catheters, guide wires, guide catheters, PTCA balloon catheters (for percutaneous transluminal coronary angioplasty) and stents in blood vessels. In-dwelling sheaths (venous and arterial), intraaortic balloon pump catheters, tubes in heart lung machines, GORE-TEX surgical prosthetic conduits and in-dwelling urethral catheters are other examples. There are, however, complications which can arise from these medical procedures. For example, the insertion of synthetic materials into lumen can cause scaring and restenosis, which can result in occlusion or blockage of the lumen. Synthetic materials in the blood vessels can also cause platelet aggregation, resulting in some instances, in potentially life-threatening thrombus formation.

Nitric oxide (referred to herein as "NO") inhibits the aggregation of platelets. NO also reduces smooth muscle proliferation, which is known to reduce restenosis. Consequently, NO can be used to prevent and/or treat the complications such as restenosis and thrombus formation when delivered to treatment sites inside an individual that have come in contact with synthetic medical devices. In addition, NO is anti-inflammatory, which would be of value for in-dwelling urethral or TPN catheters.

There are, however, many shortcomings associated with present methods of delivering NO to treatment sites. NO itself is too reactive to be used without some means of stabilizing the molecule until it reaches the treatment site. Thus, NO is generally delivered to treatment sites

in an individual by means of polymers and small molecules which release NO. However, these polymers and small molecules typically release NO rapidly. As a result, they have short shelf lives and rapidly lose their ability to deliver NO under physiological conditions. For example, the lifetime of *S*-nitroso-D,L-penicillamine and *S*-nitrosocysteine in physiological solution is no more than about an hour. As a result of the rapid rate of NO release by these compositions, it is difficult to deliver sufficient quantities of NO to a treatment site for extended periods of time or to control the amount of NO delivered.

Polymers containing groups capable of delivering NO, for example polymers containing diazeniumdiolate groups (NONOate groups), have been used to coat medical devices. However, decomposition products of NONOates under oxygenated conditions can include nitrosamines (Ragsdale *et al., Inorg. Chem.* *4*:420 (1965), some of which may be carcinogenic. In addition, NONOates generally release NO·, which is rapidly consumed by hemoglobin and can be toxic in individuals with arteriosclerosis. Further, the elasticity of known NO-delivering polymers is generally inadequate, making it difficult to coat medical devices with the polymer and deliver NO with the coated device under physiological conditions. Protein based polymers have a high solubility in blood, which results in short lifetimes. Finally, many NO-delivering polymers cannot be sterilized without loss of NO from the polymer and amounts of NO delivered are limiting.

There is, therefore, a need for new compositions capable of delivering NO to treatment sites in a manner which overcomes the aforementioned shortcomings.

Summary of the Invention

The present invention relates to novel polymers derivatized with NO_X, wherein X is one or two. It has now been found that medical devices coated with the novel polymers of the present invention are effective in reducing platelet deposition and restenosis when implanted into animal models. Specifically, stents coated with an *S*-nitrosylated β-cyclodextrin or an *S*-nitrosylated β-cyclodextrin complexed with *S*-nitroso-*N*-acetyl-D,L-penicillamine or *S*-nitroso-penicillamine resulted in decreased platelet deposition when inserted into the coronary or cortoid arteries of dogs compared with stents which lacked the polymer coating (Example 12). It has also been found that *S*-nitrosylated β-cyclodextrin and *S*-nitrosylated β-cyclodextrin complexed with *S*-nitroso-*N*-acetyl-D,L-penicillamine cause vasodilation in bioassays (Examples 8 and 10). Furthermore, compositions comprising *S*-nitrosylated cyclodextrins complexed with *S*-nitrosothiols have been found to deliver NO-related activity for extended periods of time and to exhibit increased shelf stability compared with compounds presently used to deliver NO in vivo. Specifically, *S*-nitrosylated β-cyclodextrin complexed with *S*-nitroso-*N*-acetyl-D,L-penicillamine can be stored for at least three weeks without losing NO and to deliver NO in physiological solutions for periods of time greater than 24 hours (Example 10). Lifetimes of many months have been observed (Examples 9 and 10).

The present invention includes novel nitrated or nitrosylated polymers. Thus, the novel polymers are derivatized with NO_X. The polymer has at least one NO_X group per 1200 atomic mass units (amu) of the polymer, preferably per 600 amu of the polymer, and even more

preferably per 70 amu of the polymer. In a preferred embodiment, the polymer has pendant -S-NO and/or pendant -O-NO groups, i.e. the polymer is *S*-nitrosylated and/or *O*-nitrosylated. In another embodiment, the polymer is prepared by reacting a polythiolated polysaccharide with a nitrosylating agent or a nitrating agent under conditions suitable for nitrosylating or nitrating free thiol groups.

Another embodiment of the present invention is a method of preparing a polymer having NO_x groups. The method comprises reacting a polymer having a multiplicity of pendant nucleophilic groups with a nitrosylating agent or a nitrating agent under conditions suitable for nitrosylating or nitrating free nucleophilic groups. In a preferred embodiment, the polymer is a polythiolated polymer.

Another embodiment of the present invention is a method of delivering nitric oxide to a treatment site in an individual or animal. The method comprises providing a medical device coated with a polymer derivatized with NO_x, as described above. Preferably, the polymer is an *S*-nitrosylated polymer. The medical device is then implanted into the individual or animal at the treatment site. For delivering nitric oxide to a bodily fluid, for example blood, the bodily fluid is contacted with the coated medical device.

Yet another embodiment of the present invention is a method of preparing a device for delivering nitric oxide to a treatment site in an individual or animal. The method comprises coating a medical device suitable for contacting the treatment site in the individual or animal with a polymer derivatized with NO_x, as described above. Preferably, the polymer is an *S*-nitrosylated polymer.

Another embodiment of the present invention is a medical device for delivering nitric oxide to a treatment

site in an individual or animal. The device comprises a medical device suitable for implantation at the treatment site in the individual or animal and which is coated with a polymer derivatized with NO_x, as described above. Preferably, the polymer is an *S*-nitrosylated polymer.

Another embodiment of the present invention is a method for replacing a loss of NO groups from an *S*-nitrosylated polymer. The method comprises contacting the *S*-nitrosylated polymer with an effective amount of a gaseous nitrosylating agent such as nitrosyl chloride (NOCl) under conditions suitable for nitrosylating free thiols.

S-nitrosylated cyclodextrins of the present invention undergo heterolytic cleavage of the -S-NO group, and consequently do not principly release NO. These polymers have a high NO capacity and incorporation of nitrosylating agents such as *S*-nitroso-*N*-acetyl-D,L-penicillamine into the polymer matrix increases the stability of *S*-nitrosylated cyclodextrins to weeks or more. The incorporation of nitrosylating agents also increases their capacity to deliver NO by about two fold over native cyclodextrin and by about two hundred fold over protein based polymers. The combination of increased stability and capacity to deliver NO results in a high NO potency, a controlled delivery of NO and extended treatment and storage lives for the polymer. A further advantage of these polymers is that they lack the brittleness of other NO-delivering compositions and have sufficient elasticity to coat and adhere under physiological conditions to medical devices such as stents.

Brief Description of the Figure

Figure 1 is a graph illustrating the number of platelets deposited per square centimeter on stents coated with *S*-nitrosylated β-cyclodextrin and on uncoated control stents which had been implanted in the arteries of dogs.

Figure 2 is a graph illustrating the number of -S-NO groups per cyclodextrin on the product resulting from the reaction of per-6-thio-β-cyclodextrin with one (1X), two (2X), three (3X), six (6X) and ten (10X) equivalents of acidic nitrite.

Figure 3 is the visible/ultraviolet spectrum of a reaction mixture comprising β-cyclodextrin and a 50 fold excess of acidic nitrite, taken at intervals of (1) 5 minutes, (2) fifteen minutes, (3) thirty minutes, (4) forty-five minutes, (5) sixty minutes, (6) seventy five minutes and (7) ninety minutes.

Detailed Description of the Invention

As used herein "polymer" has the meaning commonly afforded the term. Example are homopolymers, co-polymers (including block copolymers and graft copolymers), dendritic polymers, crosslinked polymers and the like. Suitable polymers include synthetic and natural polymers (e.g. polysaccharides, peptides) as well as polymers prepared by condensation, addition and ring opening polymerizations. Also included are rubbers, fibers and plastics. Polymers can be hydrophilic, amphiphilic or hydrophobic. In one aspect, the polymers of the present invention are non-peptide polymers.

Preferred polymers are those which are water insoluble an hydrophilic, i.e. can form hydrogels. A hydrogel is a composition which can absorb large quantities of water. Polymers which can form hydrogels are generally more

biocompatible than other polymers and can be used in devices which are inserted into, for example, vascular systems. Platelets and proteins normally deposit immediately upon insertion of polymer into a vascular site and initiate a cascade of events leading to restenosis or injury. This process is slowed or eliminated with polymers that form hydrogels, resulting in reduced risk of protein deposition and platelet activation. Polymers which form hydrogels are typically crosslinked hydrophilic polymers. Further descriptions and examples of hydrogels are provided in *Hydrogels and Biodegradable Polymers for Bioapplications*, editors Attenbrite, Huang and Park, *ACS Symposium Series*, No. 627 (1996), U.S. Patent Nos. 5,476,654, 5,498,613 and 5,487,898, the teachings of which are incorporated herein by reference. Examples of hydrogels include polyethylene hydroxides, polysaccharides and crosslinked polysaccharides.

NO_x is connected to the polymers of the present invention by a single covalent bond between the nitrogen atom of NO_x and a linking group M, which is pendant, or covalently bonded to the polymer. Thus, the polymers of the present invention have pendant -M-NO_x groups. Examples of -M-NO_x groups include -S-NO_x, -O-NO_x, -NR-NO_x, -CH_2-NO_x, -NOH-NO_x, -CO-NR-NO_x, -NH-C(NH_2)=N-NO_x, =N-NR-NO_x, =N-NO_x, and >N-NO_x. Also included are aliphatic and aromatic *C*-nitro and *C*-nitroso compounds. R is -H, alkyl or substituted alkyl. Alkyl groups can be straight chained or branched and have from about one to about ten carbon atoms. Suitable substituents include -CN, halogen, phenyl and alkyl. The rate of NO delivery can be varied according to the stability of the pendant -M-NO_x group, with the less stable groups having a faster rate of NO delivery than more stable groups. -S-NO_x groups are generally the least stable, while -C-NO_x groups are generally the most stable.

$-O-NO_x$ are generally more stable than $-S-NO_x$ groups, while $-N-NO_x$ groups are generally of intermediate stability.

In a preferred embodiment, the polymers of the present invention have pendant $-S-NO_x$ groups, more preferably -S-NO groups. A polymer with -S-NO groups is referred to as an *S*-nitrosylated polymer. An "-S-NO group" is also referred to as a sulfonyl nitrite, a thionitrous acid ester, an *S*-nitrosothiol or a thionitrite. In one aspect, the *S*-nitrosylated polymer also has pendant $-O-NO_x$ groups, preferably -O-NO groups. An "-O-NO" group is referred to as a nitrite. The *S*-nitrosylated polymers of the present invention have at least one NO group per 1200 atomic mass unit of the polymer. For example, an *S*-nitrosylated polymer with a molecular weight of about 600,000 atomic mass units (amu) including the -S-NO groups would have about 500 NO groups covalently bonded to the polymer. Preferably, the *S*-nitrosylated polymers of the present invention have at least one NO group per 600 amu of the polymer (See Example 13), and, even more preferably, at least one NO group per 70 amu of the polymer (See Example 14).

A polymer with pendant-$S-NO_2$ groups is referred to as an *S*-nitrated polymer. An "$-S-NO_2$ group" is also referred to as a sulfonyl nitrate, an *S*-nitrothiol or a thionitrate. $-S-NO_2$ groups decompose *in vivo*, resulting in the delivery of NO. In one aspect, an *S*-nitrated polymer also has pendant $-O-NO_x$ groups. The *S*-nitrated polymers of the present invention have at least one NO_2 group per 1200 atomic mass unit of the polymer. Preferably, the *S*-nitrated polymers of the present invention have as least one NO_2 group per 600 amu of the polymer, and, even more

preferably, at least one NO_2 group per 70 amu of the polymer.

The polymers of the present invention can be prepared from polymers having a multiplicity of nucleophilic groups. Suitable nucleophilic groups include amines, thiols, hydroxyls, hydroxylamines, hydrazines, amides, guanadines, imines, aromatic rings and nucleophilic carbon atoms. To prepare a nitrosylated polymer, a polymer with a multiplicity of pendant nucleophilic groups is reacted with a nitrosylating agent under conditions suitable for nitrosylating the nucleophilic groups. To prepare a nitrated polymer, a polymer with a multiplicity of pendant nucleophilic groups is reacted with a nitrating agent under conditions suitable for nitrating the nucleophilic groups.

The preparation of nitrated and nitrosylated polymers will now be described with respect to *S*-nitrosylated and *S*-nitrated polymers. It should be understood that the procedures described herein for the preparation *S*-nitrosylated and *S*-nitrated polymers can be used for the nitration or nitrosylation of polymers with pendant nucleophilic groups other than thiols, as described above. Although some variation in conditions may be required, such modification can be determined by one of ordinary skill in the art with no more than routine experimentation.

S-nitrosylated polymers and *S*-nitrated polymers can be prepared from polymers having a multiplicity of pendant thiol groups, referred to herein as "polythiolated polymers". To prepare an *S*-nitrosylated polymer, a polythiolated polymer is reacted with a nitrosylating agent under conditions suitable for nitrosylating free thiol groups. To prepare an *S*-nitrated polymer, a polythiolated polymer is reacted with a nitrating agent under conditions suitable for nitrating free thiol groups. Suitable

nitrosylating agents and nitrating agents are disclosed in Feelisch and Stamler, "Donors of Nitrogen Oxides", *Methods in Nitric Oxide Research* edited by Feelisch and Stamler, (John Wiley & Sons) (1996), the teachings of which are hereby incorporated into this application. Suitable nitrosylating agents include acidic nitrite, nitrosyl chloride, compounds comprising an *S*-nitroso group (*S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), *S*-nitrosoglutathione (SNOG), *N*-acetyl-*S*-nitrosopenicillaminyl-*S*-nitrosopenicillamine, *S*-nitrosocysteine, *S*-nitrosothioglycerol, *S*-nitrosodithiothreitol and *S*-nitrosomercaptoethanol), an organic nitrite (e.g. ethyl nitrite, isobutyl nitrite, and amyl nitrite) peroxynitrites, nitrosonium salts (e.g. nitrosyl hydrogen sulfate), oxadiazoles (e.g. 4-phenyl-3-furoxancarbonitrile) and the like. Suitable nitrating agents include organic nitrates (e.g. nitroglycerin, isosorbide dinitrate, isosorbide 5-mononitrate, isobutyl nitrate and isopentyl nitrate), nitronium salts (e.g. nitronium tetrafluoroborate), and the like.

Nitrosylation with acidic nitrite can be, for example, carried out in an aqueous solution with a nitrite salt, e.g. $NaNO_2$, KNO_2, $LiNO_2$ and the like, in the presence of an acid, e.g. HCl, acetic acid, H_3PO_4 and the like, at a temperature from about -20°C to about 50°C, preferably at ambient temperature. Generally, from about 0.8 to about 2.0, preferably about 0.9 to about 1.1 equivalents of nitrosylating agent are used per thiol being nitrosylated. Sufficient acid is added to convert all of the nitrite salt to nitrous acid. Specific conditions for nitrosylating a polythiolated cyclodextrin with acidic nitrite are provided in Example 3.

Nitrosylation with NOCl can be carried out, for example, in an aprotic polar solvent such as dimethylformamide or dimethylsulfoxide at a temperature from about -20°C to about 50°C, preferably at ambient temperature. NOCl is bubbled through the solution to nitrosylate the free thiol groups. Specific conditions for nitrosylating a polythiolated cyclodextrin with NOCl are provided in Example 4.

The quantity of -*S*-NO groups present in the composition can be determined by the method of Saville disclosed in "Preparation and Detection of S-Nitrosothiols," *Methods in Nitric Oxide Research*, edited by Feelisch and Stamler, (John Wiley & Sons) pages 521-541, (1996). To calculate the amount of NO per molecular weight of polymer, the polymer concentration, e.g. carbohydrate concentration, is also determined. Carbohydrate concentration can be determined by the method disclosed in Dubois *et al.*, *Anal. Chem. 28*:350 (1956).

Polythiolated polymers can be formed from polymers having a multiplicity of pendant nucleophilic groups, such as alcohols or amines. The pendant nucleophilic groups can be converted to pendant thiol groups by methods known in the art and disclosed in Gaddell and Defaye, Angew. Chem. Int. Ed. Engl. 30:78 (1991) and Rojas *et al.*, *J. Am. Chem. Soc. 117*:336 (1995), the teachings of which are hereby incorporated into this application by reference.

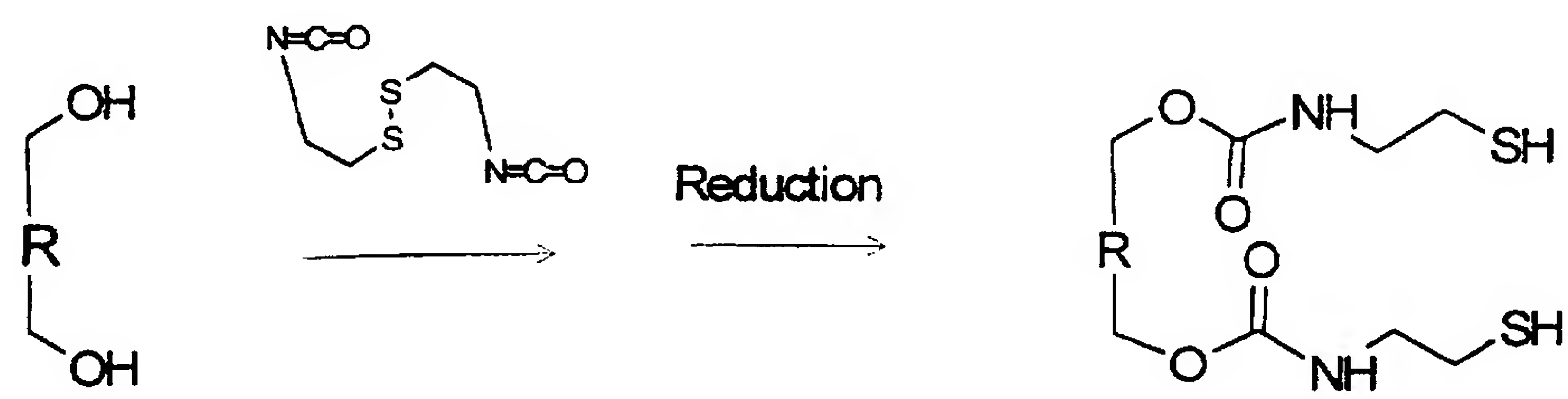
In an especially preferred embodiment, the *S*-nitrosylated polymer is an *S*-nitrosylated polysaccharide. Examples of suitable *S*-nitrosylated polysaccharides include *S*-nitrosylated alginic acid, κ-carrageenan, starch, cellulose, fucoidin, cyclodextrins such as α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin. Other suitable examples are disclosed in *Bioactive Carbohydrates*, Kennedy and White

eds., (John Wiley Sons), Chapter 8, pages 142-182, (1983) the teachings of which are incorporated herein by reference. Polysaccharides have pendant primary and secondary alcohol groups. Consequently, *S*-nitrosylated polysaccharides can be prepared from polythiolated polysaccharides by the methods described hereinabove. Preferred polysaccharides include cyclodextrins, for example α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin. The polysaccharide is first converted to a polythiolated polysaccharide, for example, by the methods disclosed in Gaddell and Defaye and Rojas et al. In these methods primary alcohols are thiolated preferentially over secondary alcohols. Preferably, a sufficient excess of thiolating reagent is used to form perthiolated polysaccharides. Polysaccharides are "perthiolated" when all of primary alcohols have been converted to thiol groups. Specific conditions for perthiolating β-cyclodextrin are given in Examples 1 and 2. Polythiolated and perthiolated polysaccharides can be nitrosylated in the presence of a suitable nitrosylating agents such as acidic nitrite (Example 3) or nitrosyl chloride (Example 4), as described above.

In one aspect, an excess of acidic nitrite is used with respect to free thiol groups when preparing an *S*-nitrosylated polysaccharide, for example an *S*-nitrosylated cyclodextrin. An excess of acidic nitrite results in a polysaccharide with pendant -S-NO and -O-NO groups. The extent of *O*-nitrosylation is determined by how much of an excess of acidic nitrite is used. For example, nitrosylation of per-6-thio-β-cyclodextrin with a 50 fold excess of acidic nitrite results in a product comprising about ten moles of NO for each cyclodextrin (Example 14), or about 1 mole of NO per 140 amu. Nitrosylation of per-6-thio-β-cyclodextrin with a 100 fold excess of acidic

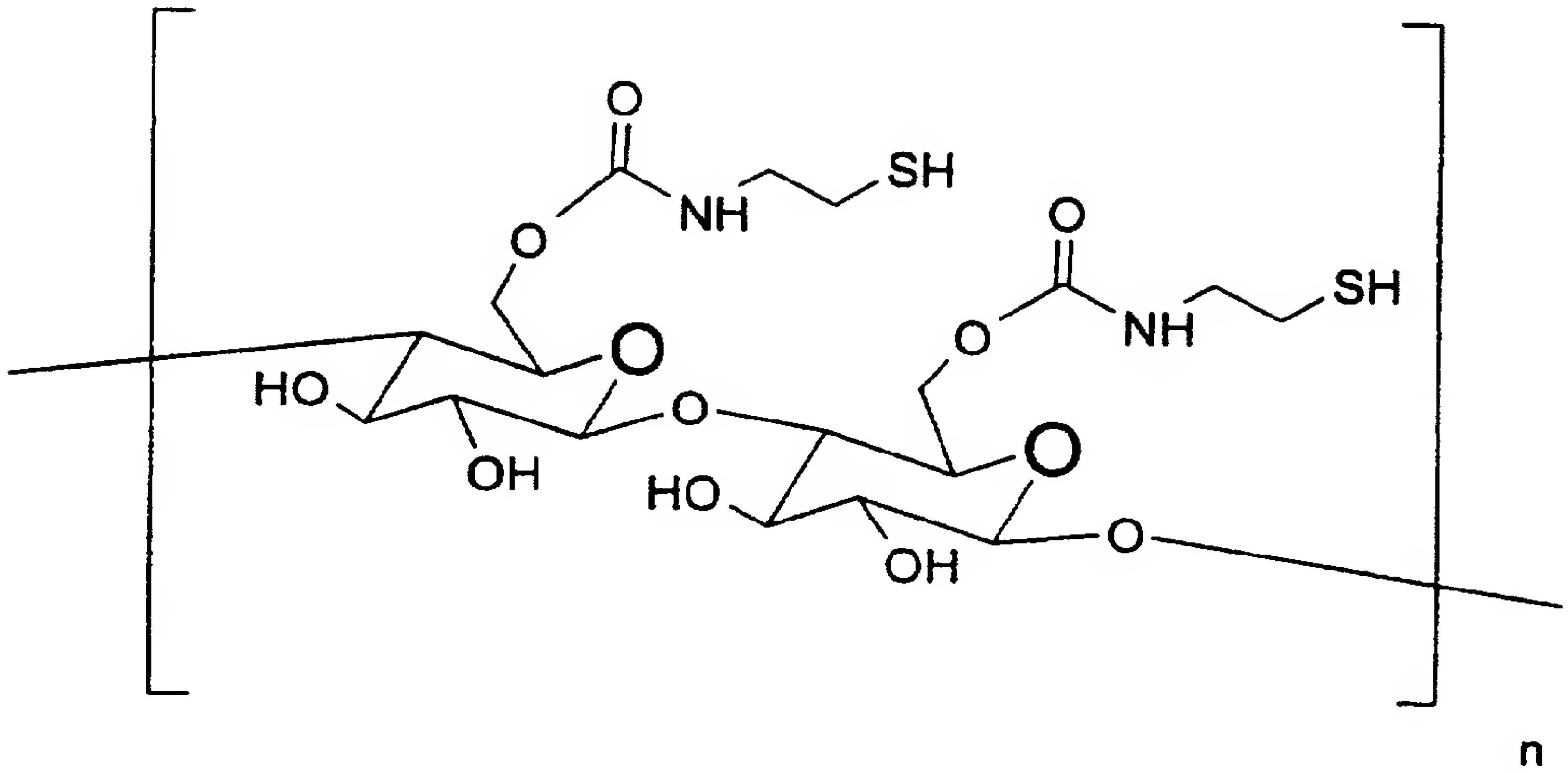
nitrite results in a product comprising about 21 moles of NO for each cyclodextrin (Example 14), or about 1 mole of NO per 70 amu. Specific conditions for the preparation of β-cyclodextrin with pendant -O-NO and -S-NO groups are described in Example 14.

In another aspect, a polythiolated polysaccharide can be prepared by reacting the alcohol groups, preferably the primary alcohol groups, on the polysaccharide with a reagent which adds a moiety containing a free thiol or protected thiol to the alcohol. In one example the polysaccharide is reacted with a bis isocyanatoalkyldisulfide followed by reduction to functionalize the alcohol as shown in Structural Formula (I):



(I)

Conditions for carrying out this reaction are found in *Cellulose and its Derivatives*, Fukamota, Yamada and Tonami, eds. (John Wiley & Sons), Chapter 40, (1985) the teachings of which are incorporated herein by reference. One example of a polythiolated polysaccharide which can be obtained by this route is shown in Structural Formula (II):



(II)

It is to be understood that agents capable of nitrosylating a free thiol, in some instances, also oxidize free thiols to form disulfide bonds. Thus, treating a polythiolated polymer (e.g. polythiolated polysaccharides such as polythiolated cyclodextrins) with a nitrosylating agent, e.g. acidified nitrite, nitrosyl chloride, *S*-nitrosothiols can, in some instances, result in the formation of a crosslinked *S*-nitrosylated polymer matrix. A "polymer matrix" is a molecule comprising a multiplicity of individual polymers connected or "crosslinked" by intermolecular bonds. Thus, in some instances the nitrosylating agent nitrosylates some of the thiols and, in addition, crosslinks the individual polymers by causing the formation of intermolecular disulfide bonds. Such polymer matrices are encompassed by the term "*S*-nitrosylated polymer" and are included within the scope of the present

invention. When an excess of the nitrosylating agent is used and when the nitrosylating agent is of a sufficient size, it can be incorporated, or "entwined," within the polymeric matrix by the intermolecular disulfide bonds which crosslink the individual polymer molecules, thereby forming a complex between the polymer and the nitrosylating agent.

S-nitrosylated polysaccharides, in particular *S*-nitrosylated cyclized polysaccharides such as *S*-nitrosylated cyclodextrins, can form a complex with a suitable nitrosylating agent when more than one equivalent of nitrosylating agent with respect to free thiols in the polythiolated polysaccharide is used during the nitrosylation reaction, as described above. Generally, between about 1.1 to about 5.0 equivalents of nitrosylating agent are used to form a complex, preferably between about 1.1 to about 2.0 equivalents.

Nitrosylating agents which can complex with an *S*-nitrosylated cyclic polysaccharide include those with the size and hydrophobicity necessary to form an inclusion complex with the cyclic polysaccharide. An "inclusion complex" is a complex between a cyclic polysaccharide such as a cyclodextrin and a small molecule such that the small molecule is situated within the cavity of the cyclic polysaccharide. The sizes of the cavities of cyclic polysaccharides such as cyclodextrins, and methods of choosing appropriate molecules for the preparation of inclusion complexes are well known in the art and can be found, for example, in Szejtli *Cyclodextrins In Pharmaceutical*, Kluwer Academic Publishers, pages 186-307, (1988) the teachings of which are incorporated herein by reference.

Nitrosylating agents which can complex with an *S*-nitrosylated cyclic polysaccharide also include nitrosylating agents with a sufficient size such that the nitrosylating agent can become incorporated into the structure of the polymer matrix of an *S*-nitrosylated polysaccharide. As discussed earlier, in certain instances nitrosylation of polythiolated polymers can also result in the crosslinking of individual polymer molecules by the formation of intermolecular disulfide bonds to give a polymer matrix. Suitable nitrosylating agents are those of an appropriated size such that the nitrosylating agent can be incorporated into this matrix. It is to be understood that the size requirements are determined by the structure of each individual polythiolated polymer, and that suitable nitrosylating agents can be routinely determined by the skilled artisan according to the particular *S*-nitrosylated polymer being prepared.

Nitrosylating agents which can form a complex with *S*-nitrosylated cyclodextrins include compounds with an *S*-nitroso group (*S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), *S*-nitrosoglutathione (SNOG), *N*-acetyl-*S*-nitrosopenicillaminyl-*S*-nitrosopenicillamine, *S*-nitrosocysteine, *S*-nitrosothioglycerol, *S*-nitrosodithiothreitol, and *S*-nitrosomercaptoethanol), an organic nitrite (e.g. ethyl nitrite, isobutyl nitrite, and amyl nitrite), oxadiazoles (e.g. 4-phenyl-3-furoxancarbonitrile), peroxynitrites, nitrosonium salts and nitroprusside and other metal nitrosyl complexes (See Feelisch and Stamler, "Donors of Nitrogen Oxides," *Methods in Nitric Oxide Research* edited by Feelisch and Stamler, (John Wiley & Sons) (1996). As discussed in greater detail below, NO delivery times and delivery capacity of *S*-

nitrosylated cyclodextrins are increased by the incorporation of nitrosylating agents. The extent and degree to which delivery times and capacity are increased is dependent on the nitrosylating agent.

Specific conditions for forming a complex between an *S*-nitrosylated cyclodextrin and a nitrosylating agent are provided in Examples 5 and 6. Conditions described in these examples result in nitrosylation of at least some of the free thiols in the polysaccharide. Because an excess of nitrosylating agent is used with respect to the quantity of free thiols in the polysaccharide is used, the resulting composition contains unreacted nitrosylating agent. Evidence that the *S*-nitrosylated polysaccharide forms a complex with the nitrosylating agent comes from the discovery, reported herein, that the rate of NO release from the reaction product of per-(6-deoxy-6--thio)β-thiocyclodextrin and *S*-nitroso-*N*-acetylpenicillamine is extended compared with *S*-nitroso-*N*-acetylpenicillamine alone (Example 10).

Although Applicants do not wish to be bound by any particular mechanism, it is believed that incorporation of a nitrosylating agent into the *S*-nitrosylated cyclic polysaccharide allows both the polysaccharide and the nitrosylating agent to deliver NO at a treatment site. It is also believed that the interaction between the cyclic polysaccharide and the nitrosylating agent results in stabilization of the -S-NO functional group in the nitrosylating agent. It is further believed that the presence of a nitrosylating agent in the composition serves to feed, i.e. replenish, the nitrosyl groups in the *S*-nitrosylated polysaccharide, thereby serving to extend the lifetime during which the polymer can serve as an NO donor.

The degree to which the lifetime of an *S*-nitrosylated cyclic polysaccharide can be extended is determined by the stability of the *S*-nitrosyl group when the nitrosylating agent is a thionitrite. The stability of -S-NO groups is dependent on a number of factors; the ability of -S-NO groups to chelate metals facilitates homolytic breakdown; tertiary -S-NO groups are more stable than secondary -S-NO groups which are more stable than primary groups; -S-NO groups which fit into the hydrophobic pocket of cyclodextrins are more stable than those which do not; the proximity of amines to the -S-NO group decreases stability; and modification at the position β to the -S-NO group regulates stability.

It is to be understood that a complex can be formed between an *S*-nitrosylated polymer or an *S*-nitrated polymer and a nitrating agent having a suitable size and hydrophobicity, as described above for *S*-nitrosylated polymers and nitrosylating agents. Crosslinked *S*-nitrated cyclodextrins and complexes between an *S*-nitrated polymer and a nitrating agent are encompassed within the term "*S*-nitrated cyclodextrin". Suitable nitrating agents include organic nitrates such as nitroglycerin, isosorbide dinitrate, isosorbide 5-mononitrate, isobutyl nitrate and isopentyl nitrate and nitronium salts. As with nitrosylating agents, the rate of NO release is dependent on which nitrating agents is incorporated into the polymer.

In one embodiment, the present invention is a composition comprising a polymer derivatized with NO_x and additionally comprising other ingredients which endow the polymer with desirable characteristics. For example, plasticizers and elastomers can be added to the composition to provide the polymer with greater elasticity. Generally, suitable plasticizers and elastomers are compounds which

are: 1) biocompatible, i.e. which cause minimal adverse reactions such as platelet and protein deposition in an individual to which it is administered and 2) which are soluble in the polymer capable of delivering NO and which can, in turn, solubilize said polymer. Examples of suitable plasticizers include polyalkylene glycols such as polyethylene glycols. Preferred plasticizers are those which can also deliver NO, for example nitrosothioglycerol.

Another embodiment of the present invention is a method of delivering NO to a treatment site in an individual or animal using the novel polymers and compositions of the present inventions to deliver NO. A "treatment site" includes a site in the body of an individual or animal in which a desirable therapeutic effect can be achieved by contacting the site with NO. An "individual" refers to a human and an animal includes veterinary animals such as dogs, cats and the like and farm animals such as horses, cows, pigs and the like.

Treatment sites are found, for example, at sites within the body which develop restenosis, injury or thrombosis as a result of trauma caused by contacting the site with a synthetic material or a medical device. For example, restenosis can develop in blood vessels which have undergone coronary procedures or peripheral procedures with PTCA balloon catheters (e.g. percutaneous transluminal angioplasty). Restenosis is the development of scar tissue from about three to six months after the procedure and results in narrowing of the blood vessel. NO reduces restenosis by inhibiting platelet deposition and smooth muscle proliferation. NO also inhibits thrombosis by inhibiting platelets and can limit injury by serving as an anti-inflammatory agent.

A treatment site often develops at vascular sites which are in contact with a synthetic material or a medical device. For example, stents are often inserted into blood

vessels to prevent restenosis and re-narrowing of a blood vessel after a procedure such as angioplasty. Platelet aggregation resulting in thrombus formation is a complication which may result from the insertion of stents. NO is an antiplatelet agent and can consequently be used to lessen the risk of thrombus formation associated with the use of these medical devices. Other examples of medical devices which contact vascular sites and thereby increase the risk of thrombus formation include sheaths for veins and arteries and GORE-TEX surgical prosthetic.

Treatment sites can also develop at non-vascular sites, for example at sites where a useful therapeutic effect can be achieved by reducing an inflammatory response. Examples include the airway, the gastrointestinal tract, bladder, uterine and corpus cavernosum. Thus, the compositions, methods and devices of the present invention can be used to treat respiratory disorders, gastrointestinal disorders, urological dysfunction, impotence, uterine dysfunction and premature labor. NO delivery at a treatment site can also result in smooth muscle relaxation to facilitate insertion of a medical device, for example in procedures such as bronchoscopy, endoscopy, laparoscopy and cystoscopy. Delivery of NO can also be used to prevent cerebral vasospasms post hemorrhage and to treat bladder irritability, urethral strictures and biliary spasms.

Treatment sites can also develop external to the body in medical devices used to treat bodily fluids temporarily removed from body for treatment, for example blood. Examples include conduit tubes within heart lung machines and tubes of a dialysis apparatus.

The method of delivering NO to a treatment site in an individual or animal comprises implanting a medical device coated with a polymer of the present invention at the treatment site. NO can be delivered to bodily fluids, for

example blood, by contacting the bodily fluid with a medical device coated with a polymer of the present invention. A preferred polymer is an *S*-nitrosylated polymer, as defined above. Examples of treatment sites in an individual or animal, medical devices suitable for implementation at the treatment sites and medical devices suitable for contacting bodily fluids such as blood are described in the paragraphs hereinabove.

"Implanting a medical device at a treatment site" refers to bringing the medical device into actual physical contact with the treatment site or, in the alternative, bringing the medical device into close enough proximity to the treatment site so that NO released from the medical device comes into physical contact with the treatment site. A bodily fluid is contacted with a medical device coated with a polymer of the present invention when, for example, the bodily fluid is temporarily removed from the body for treatment by the medical device, and the polymer coating is an interface between the bodily fluid and the medical device. Examples include the removal of blood for dialysis or by heart lung machines.

In one embodiment of the present invention, a medical device, for example a stent, is coated with a polymer of the present invention. In one example, the device is coated with an *S*-nitrosylated polysaccharide, preferably a cyclic *S*-nitrosylated or *S*-nitrated polysaccharide, and even more preferably an *S*-nitrosylated or an *S*-nitrated cyclodextrin. A mixture is formed by combining a solution comprising a polythiolated polysaccharide with a medical device insoluble in the solution. The mixture is then combined with a nitrosylating agent (or nitrosating agent) under conditions suitable for nitrosylating (or nitrating) free thiol groups, resulting in formation of an *S*-nitrosylated

polysaccharide. In an aqueous solution, the *S*-nitrosylated polysaccharide precipitates from the solution and coats the medical device. In polar aprotic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO), the medical device can be dipped into the reaction mixture and then dried *in vacuo* or under a stream of an inert gas such as nitrogen or argon, thereby coating the medical device. Suitable nitrosylating agents include acidified nitrite, *S*-nitrosothiols, organic nitrite, nitrosyl chloride, oxadiazoles, nitroprusside and other metal nitrosyl complexes, peroxynitrites, nitrosonium salts (e.g. nitrosyl hydrogensulfate) and the like. Suitable nitrating agents include organic nitrates, nitronium salts (e.g. nitronium tetrafluoroborate) and the like. The polymers of the present invention are not brittle, and consequently remain adhered to the medical device, even under physiological conditions. Thus, these polymers are particularly suited for coating devices which are to be implanted in patients for extended periods of time.

It is to be understood that other methods of applying polymer coatings to devices, including methods known in the art, can be used to coat medical devices with the polymers of the present invention.

Another embodiment of the present invention is a method of replacing a loss of NO groups from an *S*-nitrosylated polymer. As discussed above, NO is lost from *S*-nitrosylated compounds over time. In addition, sterilization of medical instruments containing *S*-nitrosylated compounds also results in the loss of NO from *S*-nitrosylated compounds. The loss of NO from *S*-nitrosylated compounds reduces the capacity of the compound to deliver NO to a treatment site. NO groups can be replaced by contacting the *S*-nitrosylated polymer with an

effective amount of a gaseous, nitrosylating agent such as nitrosyl chloride or nitric oxide.

An "effective amount" of a gaseous, nitrosylating agent is the quantity which results in nitrosylation free thiol groups in the compound or polymer. Preferably, a sufficient amount of the gaseous, nitrosylating agent is used to saturate the free thiol groups in the compound or polymer with NO, i.e. all of the thiol groups become nitrosylated. An effective amount ranges from about 0.8 atmospheres to about 10 atmospheres and is preferably about one atmosphere.

Another embodiment of the present invention is a method of replacing a loss of NO or NO_2 groups from a nitrated or nitrosylated polymer at a treatment in an individual. The method comprises administering to the individual a regenerating amount of a nitrating agent or a nitrosylating agent suitable for regenerations pendant nucleophilic groups with NO_2 or NO groups, as described above. Examples include *S*-nitrosothiols, organic nitrites, oxadiazoles, metal nitrosyl complexes, organic nitrates, peroxynitrites, nitrosonium salts and nitronium tetrafluoroborate. Although Applicants do not wish to be bound by any particular mechanism, it is believed that some of the nitrating agent or nitrosylating agent will contact the polymer at the treatment site and nitrate or nitrosylate the free nucleophilic groups *in vivo*, thereby regenerating the NO_2 or NO capacity of the polymer.

A "regenerating amount" of a nitrating or nitrosylating agent is an amount which results in a sufficiently high local concentration of the agent at a treatment site to nitrate or nitrosylate the free pendant nucleophilic groups of a polymer located at the treatment site. A "regenerating amount" is also an amount which does not cause undue undesirable side effects in the individual.

It will be understood that the amount administered to the individual will depend on factors such as the age, weight, sex and general health of the individual, and that the skilled person will be able to vary the amount administered, taking such factors into account. For example, dosages can be from about 10 mg/kg/day to about 1000 mg/kg/day. The compound can be administered by an appropriate route in a single dose or multiple doses.

A variety of routes of administration are possible including, but not necessarily limited to parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), oral (e.g., dietary), nasal, slow releasing microcarriers, or rectal, depending on the disease or condition to be treated. Oral, parenteral and intravenous administration are preferred modes of administration. Formulation of the compound to be administered will vary according to the route of administration selected (e.g., solution, emulsions, aerosols, capsule). An appropriate composition comprising the compound to be administered can be prepared in a physiologically acceptable vehicle or carrier. For solutions or emulsions, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles can include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replenishers (See, generally, Remington's Pharmaceutical Science, 16th Edition, Mack, Ed. (1980)).

The invention is further illustrated by the following examples, which are not intended to be limiting in any way.

Example 1

Preparation of Per-(6-deoxy-6-iodo)-β-iodocyclodextrin

β-Cyclodextrin (20.0 g, 17.6 mmol, 123 mmol primary hydroxyl) was added to a stirred solution of triphenylphosphine (97.2 g, 371 mmol, 3 eq per primary hydroxyl) and iodine chips (93.5 g, 371 mmol, 3 eq per primary hydroxyl) in dimethylformamide (DMF) (400 mL); the mixture warmed on addition. The solution was placed in an oil bath at 80°C for 20 hours, then permitted to cool to room temperature DMF (350 mL) was removed under reduced pressure to yield a thick, the dark syrup was roughly one-third the volume of the original solution. To this syrup, cooled in an ice bath, was added 160 mL of 3 M NaOMe; the pH was found to be 9 (pH paper with a drop of water). After addition, the syrup was permitted to warm to room temperature and stirred for an additional 1 hour. The syrup was then poured into MeOH (3600 mL) to give a small amount of precipitate. Water (1000 mL) was added slowly to the MeOH solution, yielding a milky white precipitate in the dark brown solution. The precipitate was removed by filtration to give a yellow solid that was washed several times with MeOH (1000 mL total) to remove most of the color, giving a tan solid that was Soxhlett-extracted for >12 hours and dried under high vacuum to give 19.84 of an off-white solid (59%).

Example 2

Preparation of Per-(6-deoxy-6--thio)β-thiocyclodextrin

Per-(6-deoxy-6-iodo)-β-cyclodextrin (19.84 g, 10.4 mmol, 72.9 mmol primary iodide) was dissolved in DMF (210 mL) and thiourea (6.3 g, 82.8 mmol, 1.13 eq) was added. The solution was stirred at 70°C under nitrogen for 48 hours. DMF was removed under reduced pressure to give an orange oil, which was added to aqueous NaOH (5.4 g in 1000 mL, 135 mmol) to give a white precipitate on stirring. The solution was heated to a gentle reflux for 1 hour, which

effected full solvation of the precipitate, then cooled, which resulted in formation of a precipitate that was removed by filtration and washed with water (this precipitate was not used). The solution was acidified with 1 M $KHSO_4$ to give a fine white precipitate that was filtered and washed with water, then air-dried overnight. The precipitate was suspended in water (700 mL), then solvated by addition of 70 mL of aqueous 1 M NaOH, then re-precipitated with 90 mL of aqueous 1 M $KHSO_4$. The precipitate was filtered, air-dried overnight, then dried under high vacuum to give 6.0 g (46%) of an off-white solid, mp 289°C (dec).

Example 3
Nitrosylation of Per-6-thio-ß-cyclodextrin with Acidic Nitrite

Per-(6-deoxy-6-thio)-β-cyclodextrin (500 mg, 0.401 mmol, 2.81 mmol primary thiol) was dissolved in 0.5 M aqueous NaOH (10 mL) to give a faintly yellow solution. A mixture of 2.8 mL 1 M aqueous $NaNO_2$ (2.8 mmol, 1 equivalent per mole free thiol) and 2 M HCl (15 mL) was quickly added to give a brick-red precipitate. The precipitate was pelleted by centrifuge, and the acidic supernatant was removed by syringe. Deionized water was added and the precipitate was agitated to full dispersion. The centrifugation/supernatant removal process was repeated six times (until the supernatant was neutral to pH paper), giving a deep red pellet in a small amount of water.

Example 4
Nitrosylation of per-6-thio-ß-cyclodextrin with Nitrosyl Chloride in DMF

Per-(6-deoxy-6-thio)-β-cyclodextrin (50 mg, 0.04 mmol, 0.28 mmol primary thiol) was dissolved in DMF (1 mL). Nitrosyl chloride was bubbled through to give a deep brown

solution. The solvent can be removed *in vacuo* or under a stream of an inert gas such as nitrogen or argon to afford the polymer product.

Example 5
Nitrosylation of Per-6-thio-ß-cyclodextrin with *S*-Nitroso-*N*-Acetylpenicillamine

Per-(6-deoxy-6-thio)-ß-cyclodextrin (32.3 mg, 0.0259 mmol, 0.181 mmol primary thiol) was dissolved in 1 mL 1 M NaOH. D(+)-*S*-nitroso-*N*-acetylpenicillamine (57.0 mg, 1.4 eq per thiol) was added to give a deep-red precipitate. The precipitate was pelleted by centrifuge, and the acidic supernatant was removed by syringe. Deionized water was added and the precipitate was agitated to full dispersion. The centrifugation/supernatant removal process was repeated four times (until the supernatant was neutral to pH paper), giving a deep red pellet in a small amount of water.

Example 6
Nitrosylation of Per-6-thio-ß-cyclodextrin with *S*-Nitroso-*N*-Acetylpenicillamine in Dimethylformamide

Per-(6-deoxy-6-thio)-ß-cyclodextrin (10 mg, 0.0080 mmol, 0.056 mmol primary thiol) was dissolved in 1 mL DMF. D(+)-*S*-nitroso-*N*-acetylpenicillamine (17.7 mg, 0.080 mmol, 1.4 eq per thiol) was added to give a green solution. After standing for 2 hours, the solution had turned deep red. The solvent can be removed *in vacuo* or under a stream of an inert gas such as nitrogen or argon to afford the polymer product.

Example 7

Method for Assaying Nitric Oxide Release

The capacity of a compound to cause relaxation of vascular smooth muscle, measured by the degree and duration of vasodilation resulting from exposure of a blood vessel to the compound, is a measure of its ability to deliver NO *in vivo*. Methods reported in Stamler *et al.*, *Proc. Natl. Acad. Sci. USA 89*:444 (1992), Osborne *et al.*, *J. Clin. Invest. 83*:465 (1989) and the chapter by Furchgott in *Methods in Nitric Oxide Research*, edited by Feelisch and Stamler, (John Wiley & Sons) (1996), were used to measure vascular smooth muscle contraction. Because lower concentrations of NO are required to inhibit platelet aggregation than vasodilation, measurement of smooth muscle contraction provides a good indication of whether a composition delivers sufficient NO to reduce platelet aggregation.

New Zealand White female rabbits weighing 3-4 kg were anesthetized with sodium pentobarbital (30 mg/kg). Descending thoracic aorta were isolated, the vessels were cleaned of adherent tissue, and the endothelium was removed by gentle rubbing with a cotton-tipped applicator inserted into the lumen. The vessels were cut into 5-mm rings and mounted on stirrups in 20 mL organ baths. The rings were suspended under a resting force of 1 g in 7 ml of oxygenated Kreb's buffer (pH 7.5) at 37°C and allowed to equilibrate for one hour. Isometric contractions were measured on a Model 7 oscillograph recorder connected to transducers (model TO3C, Grass Instruments, Quincy, MA). Fresh Krebs solution was added to the bath periodically during the equilibration period and after each test response. Sustained contractions were induced with 7 μM norepinephrine prior to the addition of the test compound.

Example 8

Delivery of Nitric Oxide by a Polymer Coated Stent

The ability of *S*-nitrosylated β-cyclodextrin (referred to as "free polymer") to cause continuous vasodilation was compared with the NO-related activity of a stent coated with *S*-nitrosylated β-cyclodextrin. *S*-nitrosylated β-cyclodextrin was obtained by the method described in Example 3. Polymer-coated stents were obtained by suspending a stent in the reaction mixture prepared according to the procedure described in Example 3, thereby allowing the precipitated *S*-nitrosylated β-cyclodextrin to coat the stent. Alternatively, polymer-coated stents were obtained by dipping a stent into a reaction mixture prepared by the method of Example 4. In either case, the polymer-coated stent was then dried *in vacuo* or under a stream of a nitrogen. The delivery of NO by the polymer coated stent and by the free polymer was assayed according to the procedure described in Example 7.

The polymer coated stent resulted in continuous vasodilation for more than one hour. Removal of the stent resulted in immediate restoration of tone, indicative of continuous NO release.

A fresh polymer coated stent was added to the organ chamber. The stent was then removed from the organ chamber and transferred to a second organ chamber. Similar levels of smooth muscle relaxation were observed to occur in each organ chamber, which is indicative of continuous release of NO from the *S*-nitrosylated β-cyclodextrin.

Example 9

Stability of Polymers Prepared by Nitrosylating Per-6-Thio-β-Cyclodextrin with *S*-Nitroso-*N*-Acetylpenicillamine

The *S*-nitrosylated polymer prepared by the method described in Example 5 was placed on a metal base and dried *in vacuo* or under a stream of nitrogen to give a brown solid. This solid had an absorabance of about 15 in the visible range from about 540 to about 600 nanometers. Concentrations of NO in the 1.0 mM range are sufficient to give an absorbance of about 0.15 in this region of the visible spectrum.

The polymer was then stored and protected from light for three weeks. The absorbance in the region from about 540-600 nanometers was essentially unchanged, indicating retention of *S*-NO by the polymer. In addition, the ability of the compound to cause vasodilation, as measured by the assay described in Example 7, also remained essentially unchanged over the three week period.

Example 10

Incorporating *S*-Nitroso-*N*-Acetylpenicillamine Into *S*-Nitrosylated Polymers Increases the Nitric Oxide Delivering Capacity and Half-Life of the Polymers

S-Nitroso-penicillamine, *S*-nitrosylated β-cyclodextrin (prepared according to the procedure in Example 3) and *S*-nitrosylated β-cyclodextrin complexed with *S*-nitroso-penicillamine (prepared according to the procedure in Example 5) were assayed by the method described in Example 7 for their ability to cause vasodilation. In addition, the half-lives for these compositions in physiological solution were measured. The half-life is time required for the composition to lose one half of its bound NO. The

amount of NO in the composition is determined by the method of Saville, as described in Example 13.

S-nitrosylated β-cyclodextrin complexed with *S*-nitroso-penicillamine was found to deliver several orders of magnitude more NO in physiological solution than *S*-nitroso-penicillamine. In addition, *S*-nitroso-penicillamine was able to deliver NO for no more than about one hour, while *S*-nitrosylated β-cyclodextrin complexed with *S*-nitroso-penicillamine had a half-life of greater than forty hours. This result indicates that incorporating *S*-nitroso-penicillamine into the polymer matrix results in stabilization of the *S*-nitroso-penicillamine -*S*-NO group.

Incorporation of *S*-nitroso-penicillamine into the polymer matrix of *S*-nitrosylated β-cyclodextrin resulted in an extension of the time period during which nitric oxide can released. The half-life of *S*-nitrosylated β-cyclodextrin was greater than about eighteen hours, while the half-life of *S*-nitrosylated β-cyclodextrin complexed with *S*-nitroso-penicillamine was greater than about forty hours. This result indicates that it is possible to extend the time period during which *S*-nitrosylated polymers can release NO, based on the type of NO donor that is incorporated into the polymer matrix. This result also suggests that the NO donor is "empowering" the polymer with NO activity, thus serving to extend the polymer lifetime.

Example 11

Assay For Determining Antiplatelet Effects

Venous blood, anticoagulated with 3.4 mM sodium citrate was obtained from volunteers who had not consumed acetylsalicylic acid or any other platelet-active agent for at least 10 days. Platelet-rich plasma was prepared by

centrifugation at 150xg for 10 minutes at 25°C. Platelet counts were determined with a Coulter Counter (model ZM).

Aggregation of platelet-rich plasma was monitored by a standard nephelometric technique, in which 0.3-ml aliquots of platelets were incubated at 37°C and stirred at 1000 rpm in a PAP-4 aggregometer (Biodata, Hatsboro, PA).

S-Nitrosylated β-cyclodextrin, prepared according to the method described in Example 3, was incubated at concentrations of 1 μM, 10 μM and 100 μM in 400 μL of platelet rich plasma for 3 minutes. Aggregations were induced by adding 100 μL of 10 μM ADP. Controls were run in the absence of polymer. Aggregations were quantified by measuring the maximal rate and extent of change of light transmittance and are expressed as normalized value relative to control aggregations.

Dose-dependent inhibition of ADP-induced platelet aggregation was observed over the range of 1 μM to 100 μM *S*-nitrosylated β-cyclodextrin. Inhibition of platelet aggregation was observed, even at the lowest concentration.

Example 12

Inhibition of Platelet Deposition in Dogs by *S*-Nitrosylated ß-Cyclodextrin Coated Stent

Platelets play a central role in the development of acute closure as well as late restenosis following angioplasty. Potent inhibitors of the platelet glycoprotein II_B/III_A when given systemically have been shown to be effective in reducing 30 day and 6 month clinical events following high risk angioplasty. This benefit, however, has come at the expense of higher rates of bleeding complications. By delivery of a potent glycoprotein II_B/III_A inhibitor locally, the benefits of platelet inhibition may be attained without the risk of systemic platelet inhibition. The purpose of this study is

to determine the local platelet inhibitory effects of cyclodextrin-nitric oxide.

Methods

Seven mongrel dogs were studied. After diagnostic angiography, stents were implanted into the LAD and LCX arteries. The first 3 animals received plain 8 mm corrugated metal ring stents and the remaining 4 were given SNO-cyclodextrin coated stents. Coronary dimensions were obtained utilizing on-line QCA measurements and stents were appropriately sized to achieve a 1.2-13:1 stent to artery ratio. Prior to stent implantation, autologous platelets were labeled with Indium 111 oxime, reinfused and allowed to recirculate for 1 hour. The assigned stents were then deployed at 10-14 ATMs and quantitative coronary angiography was repeated. Platelets were allowed to circulate an additional 24 hours then the study was terminated for platelet deposition analysis.

Results

Platelet deposition on plain metal stents was greater than on NO coated stents although the difference was not statistically significant: 5.19 ± 5.78 vs. 4.03 ± 5.33 platelets x $10^8/cm^2$ $p=0.5827$. However, 4 of the 6 metal controls had greater platelet deposition than any of the NO coated stents. The mean for the metal controls was affected by 2 very low values. These data suggest that the drug prevents above baseline platelet deposition as was seen in 4 of the 6 metal stents without NO coating. The number of platelets/square centimeter on each of the control stents and on each of the coated stents are shown in Figure 1.

Example 13

Determination of the Amount of *S*-Nitrosylation in *S*-Nitrosylated Polysaccharides

Determination of Carbohydrate Concentration

The amount of carbohydrate present is determined by the following disclosed in Dubois *et al., Anal. Chem. 28*:350 (1956). Two milliliters of carbohydrate solution containing between 10 and 70γ of carbohydrate are pipetted into a colorimetric tube, and 0.05 ml of 80% phenol is added. Then 5 ml of concentrated sulfuric acid is added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes are allowed to stand 10 minutes, then they are shaken and placed for 10 to 20 minutes in a water bath at 25°C to 30°C before readings are taken. The color is stable for several hours and readings may be made later if necessary. The absorbance of the characteristic yellow-orange color is measured at 490 mμ of hexoses and 480 mμ for pentose and uronic acids. Blanks are prepared by substituting distilled water for sugar solution. The amount of sugar may then be determined by reference to a standard curve previously constructed for the particular sugar under examination.

All solutions are prepared in triplicate to minimize errors resulting from accidential contamination with cellulose lint.

Determination of R-S-NO Concentration

The concentration of R-S-NO groups in a sample is based on the method reported in Saville, Analyst *83*:620 (1958). By this method, R-S-NO groups are converted into an azo dye. The concentration of this dye is determined by

measuring the absorbance at 540 nm ($\epsilon \sim 50{,}000\ M^{-1}\ cm^{-1}$). The procedure is as follows:

Reagent

Solution A: sulfanilamide 1% dissolved in 0.5 M HCl.

Solution B: same solution as used in A to which 0.2% $HgCl_2$

Solution C: 0.02% solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in 0.5 M HCl.

Procedure

A given volume (50 μl-1m) of the sample to be assayed is added to an equivalent volume of solution A and solution B. The two samples are set aside for 5 minutes to allow formation of the diazonium salt, after which an equivalent volume of solution C is added to each mixture. Color formation, indicative of the azo dye product, is usually complete by 5 minutes. The sample absorbance is then read spectrophotometrically at 540 nm. The RSNO is quantified as the difference in absorbance between solution B and A. (i.e. B - A). In the event that the background nitrite concentration is high (i.e. increased background in A), the accuracy of the measurement can be increased by the addition of an equivalent volume of 0.5% ammonium sulfamate in acid (45 mM) 5 minutes prior to the addition of sulfanilamide. The nitrous acid in solution reacts immediately with excess ammonium sulfamate to form nitrogen gas and sulfate.

Concentrations of thiol greater than 500 μM in samples may interfere with the assay if nitrite is also present at micromolar concentration. Because nitrite will nitrosate indiscriminantly under the acidic conditions employed, thiols will effectively compete for reaction with sulfanilamide (present at 50 mM in this assay) as their concentration approaches the millimolar range. This will

lead to artifactual detection of RSNO. The problem can be avoided by (1) keeping the ratio of thiol to sulfanilamide < 0.01, (2) first alkylating thiols in the solution, or (3) adding free thiols to standards to correct for the potential artifact.

S-nitrosylated β-cyclodextrin was prepared as described in Example 3 using 1 mM perthiolated β-cyclodextrin and 1) one equivalent (1X); 2) two equivalents (2X); three equivalents (3X); six equivalents (6X); and ten equivalents (10X) of acidic nitrite. The carbohydrate concentration and the -S-NO concentration of each resulting carbohydrate polymer was then determined, as described above. The results are shown in Figure 2. A six fold excess of acidified nitrite results in about three -S-NO groups per molecule of cyclodextrin, or about one -S-NO group per 470 molecular weight. The use of three and ten equivalents of acidified nitrite results in a product with between about 2 and 2.5 -S-NO groups per cyclodextrin.

Example 14

Preparation of *O*- and *S*-Nitrosylated β-Cyclodextrin

β-Cyclodextrin with pendant -O-NO and -S-NO groups was prepared according to the procedure described in Example 3 except that 50 and 100 equivalents of acidic nitrite were used.

The formation of -O-NO groups is accompanied by an increase in absorbance in the 320-360 nm range of the ultraviolet/visible spectrum. Because -S-NO groups also absorb in this region of the ultraviolet/visible spectrum, confirmation of *O*-nitrosylation is provided by the observation that the increase in absorbance in the 320-360 nm region is accompanied by no further increase in the -S-NO concentration. The concentration of -S-NO is determined by the method of Saville, described in Example

13. The amount of -O-NO present in the polymer can be determined by the intensity of the absorbance in the 320-360 nm region and the loss of NO from media. The quantity of -O-NO per molecular weight can be calculated by first determining the carbohydrate concentration, as described in Example 13 above.

Figure 3 shows the ultraviolet/visible spectrum of the β-cyclodextrin in the presence of a 50 fold excess of acidic nitrite, as described above. As can be seen, the absorbance in the 340-350 nm region increases over time, with a maximum being reached after about 45 minutes. The combined concentration of -O-NO and -S-NO groups was determined to be about 10 NO groups per cyclodextrin when a 50 fold excess of acidic nitrite were used or about one NO group per 140 amu. The combined concentration of -O-NO and -S-NO groups was determined to be about 21 NO groups per cyclodextrin when a 100 fold excess of acidified nitrite were used or about one NO group per 67 amu.

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. A polymer derivatized with at least one $-NO_X$ group per 1200 atomic mass units of the polymer, wherein X is one or two.

2. The polymer of Claim 1 comprising at least one $-NO_X$ group per 600 atomic mass units of the polymer.

3. The polymer of Claim 2 wherein the polymer is hydrophilic and water insoluble.

4. The polymer of Claim 2 comprising at least one NO_X group per 70 atomic mass units of the polymer.

5. The polymer of Claim 2 wherein the polymer is *S*-nitrosylated.

6. The polymer of Claim 5 wherein the polymer is an *S*-nitrosylated polysaccharide.

7. The polymer of Claim 6 wherein the *S*-nitrosylated polysaccharide is cyclic.

8. The polymer of Claim 7 wherein the polysaccharide is an *S*-nitrosylated cyclodextrin.

9. The polymer of Claim 8 wherein the *S*-nitrosylated cyclodextrin is an *S*-nitrosylated poly-β-cyclodextrin.

10. The polymer of Claim 9 wherein the *S*-nitrosylated poly-β-cyclodextrin is poly[per-(6-deoxy-6--thio)β-thiocyclod

11. The polymer of Claim 1 wherein the polymer is an *S*-nitrated cyclic polysaccharide.

12. The polymer of Claim 7 wherein the *S*-nitrosylated cyclic polysaccharide is complexed with a nitrosylating agent or a nitrating agent.

13. The polymer of Claim 12 wherein the *S*-nitrosylated cyclic polysaccharide is an *S*-nitrosylated cyclodextrin.

14. The polymer of Claim 13 wherein the nitrosylating agent is selected from the group consisting of an *S*-nitrosothiol, an organic nitrite, an oxadiazole, a peroxynitrite, a nitrosonium salt and a metal nitrosyl complex.

15. The polymer of Claim 13 wherein the nitrosylating agent is an *S*-nitrosothiol selected from the group consisting of *S*-nitroso-*N*-acetyl-D,L-penicillamine, *S*-nitrosoglutathione, *S*-nitrosocysteine, *S*-nitrosodithiothreitol, *S*-nitrosomercaptoethanol, *S*-nitrosothioglycerol and *S*-nitrosopenicillamine.

16. The polymer of Claim 13 wherein the nitrating agent is selected from the group consisting of an organic nitrate and a nitronium salt.

17. A composition comprising an *S*-nitrosylated polymer derivatized with at least one NO_x group per 600 atomic mass units of the polymer and an elastomer or plasticizer.

18. The composition of Claim 17 wherein the plasticizer is nitrosothioglycerol.

19. A polymer prepared by reacting a solution comprising a cyclic polythiolated carbohydrate with a nitrating agent under conditions suitable for nitrating free thiol groups.

20. The polymer of Claim 19 wherein the cyclic polythiolated carbohydrate is a polythiolated cyclodextrin.

21. A polymer prepared by reacting a solution comprising a cyclic polythiolated carbohydrate with a nitrosylating agent under conditions suitable for nitrosylating free thiol groups.

22. The polymer of Claim 21 wherein the cyclic polythiolated carbohydrate is a polythiolated cyclodextrin.

23. The polymer of Claim 22 wherein the nitrosylating agent is an acidified nitrite.

24. The polymer of Claim 22 wherein the polythiolated cyclodextrin is reacted with between about 0.8 and about 2.0 molar equivalents of acidified nitrite per mole of free thiol.

25. The polymer of Claim 24 wherein the polythiolated cyclodextrin is reacted with between about 0.9 and about 1.1 molar equivalents of acidified nitrite per mole of free thiol.

26. The polymer of Claim 25 wherein the polythiolated cyclodextrin is per-6-thio-β-cyclodextrin.

27. The polymer of Claim 22 wherein the nitrosylating agent is nitrosyl chloride.

28. The polymer of Claim 22 wherein the nitrosylating agent is selected from the group consisting of an *S*-nitrosothiol, an organic nitrite, an oxadiazole, a peroxynitrite, a nitrosonium salt and a metal nitrosyl complex.

29. The polymer of Claim 28 wherein the *S*-nitrosothiol is selected from the group consisting of *S*-nitroso-*N*-acetyl-D,L-penicillamine, *S*-nitrosoglutathione, *N*-acetyl-*S*-nitrosopenicillaminyl-*S*-nitrosopenicillamine, *S*-nitrosocysteine, *S*-nitrosodithiothreitol, *S*-nitrosomercaptoethanol, *S*-nitrosothioglycerol and *S*-nitrosopenicillamine.

30. A method of preparing a polymer derivatized with $-NO_x$ groups comprising reacting a polymer having at least one pendant nucleophilic group per 1200 amu of the polymer with a nitrosylating or nitrating agent under conditions suitable for causing said derivatization.

31. The method of Claim 30 wherein the polymer is polythiolated.

32. A method of preparing an *S*-nitrosylated polymer comprising reacting a polythiolated polymer with an nitrosylating agent under conditions suitable for nitrosylating free thiol groups.

33. The method of Claim 32 wherein the polythiolated polymer is a cyclic polythiolated carbohydrate.

34. The method of Claim 33 wherein the cyclic polythiolated carbohydrate is a polythiolated cyclodextrin.

35. The method of Claim 34 wherein the nitrosylating agent is acidified nitrite.

36. The method of Claim 34 wherein the polythiolated cyclodextrin is raected with between about 0.8 and about 2.0 molar equivalents of acidified nitrite per mole of free thiol.

37. The method of Claim 36 wherein the polythiolated cyclodextrin is reacted with between about 0.9 and about 1.1 molar equivalents of acidified nitrite per mole of free thiol.

38. The method of Claim 37 wherein the polythiolated cyclodextrin is per-6-thio-β-cyclodextrin.

39. The method of Claim 34 wherein the nitrosylating agent is nitrosyl chloride.

40. The method of Claim 34 wherein the nitrosylating agent is selected from the group consisting of an *S*-nitrosothiol, an organic nitrite, an oxadiazole, a

peroxynitrite, a nitrosonium salt and a metal nitrosyl complex.

41. The method of Claim 40 wherein the *S*-nitrosothiol is selected from the group consisting of *S*-nitroso-*N*-acetyl-D,L-penicillamine, *S*-nitrosoglutathione, *N*-acetyl-*S*-nitrosopenicillaminyl-*S*-nitrosopenicillamine, *S*-nitrosocysteine, *S*-nitrosodithiothreitol, *S*-nitrosomercaptoethanol, *S*-nitrosothioglycerol and *S*-nitrosopenicillamine.

42. A method of delivering nitric oxide to a treatment site in an individual or animal comprising:
   a) providing a medical device coated with a polymer having at least one $-NO_X$ group per 1200 amu of the polymer; and
   b) implanting the medical device at the treatment site.

43. The method of Claim 42 wherein the polymer is an *S*-nitrosylated polymer.

44. A method of deliverying nitric oxide to a bodily fluid comprising:
   a) providing a medical device coated with a polymer having at least one $-NO_X$ group per 1200 amu of the polymer; and
   b) contacting the bodily fluid with the medical device.

45. The method of Claim 44 wherein the polymer is an *S*-nitrosylated polymer.

46. A method of preparing a device for delivering nitric oxide to a treatment site in an individual or animal comprising coating a medical device suitable for contacting the treatment site in the individual or animal with a polymer derivatized with at least one $-NO_x$ group per 1200 amu of the polymer.

47. The method of Claim 46 wherein the polymer is an *S*-nitrosylated polymer.

48. A medical device for implantation in an individual or animal or for contacting the bodily fluid of an individual or animal, in combination with a coating on a surface of said medical device, said coating comprising a polymer derivatized with at least one $-NO_x$ group per 1200 amu of the polymer.

49. The medical device of Claim 48 wherein the medical device is a stent.

50. The medical device of Claim 48 wherein the polymer is an *S*-nitrosylated polymer.

51. A method for replacing a loss of NO groups from an *S*-nitrosylated polymer comprising contacting the *S*-nitrosylated polymer with an effective amount of a gaseous nitrosylating agent.

52. The method of Claim 51 wherein the gaseous nitrosylating agent is nitrosyl chloride or nitric oxide.

53. The method of Claim 51 wherein the polymer coats a medical device suitable for implementation at a site

requiring treatment with NO in an individual or animal.

54. The method of Claim 53 wherein the loss of NO results from sterilization of the medical device.

55. A method for replacing a loss of NO_2 or NO groups from a polymer having pendant nitrated or nitrosylated nucleophilic groups at a treatment in an individual comprising administering to the individual with a regenerating amount of a nitrating agent or a nitrosylating agent.

PLATELET DEPOSITION ON STENTS IMPLANTED IN DOGS

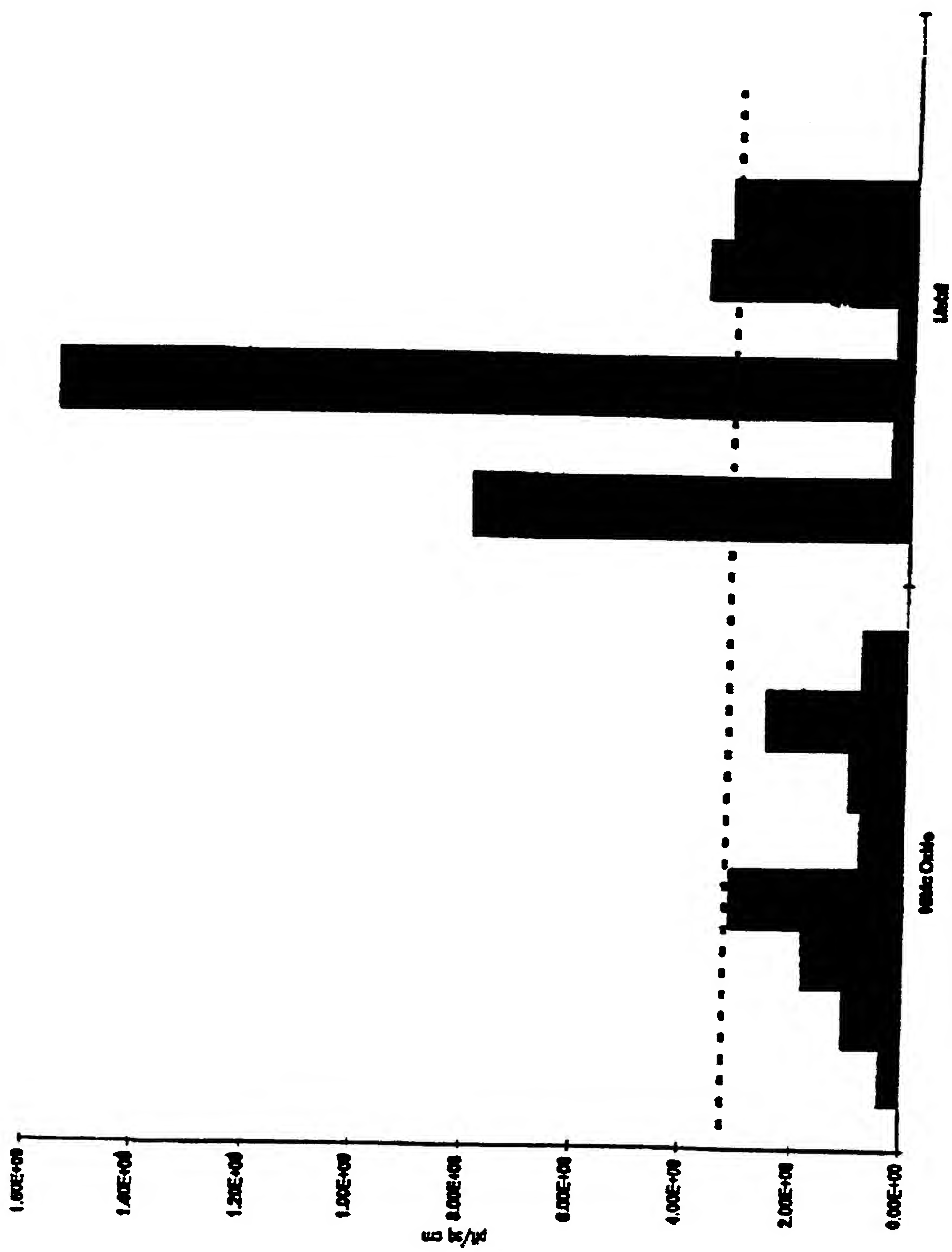


Figure 1

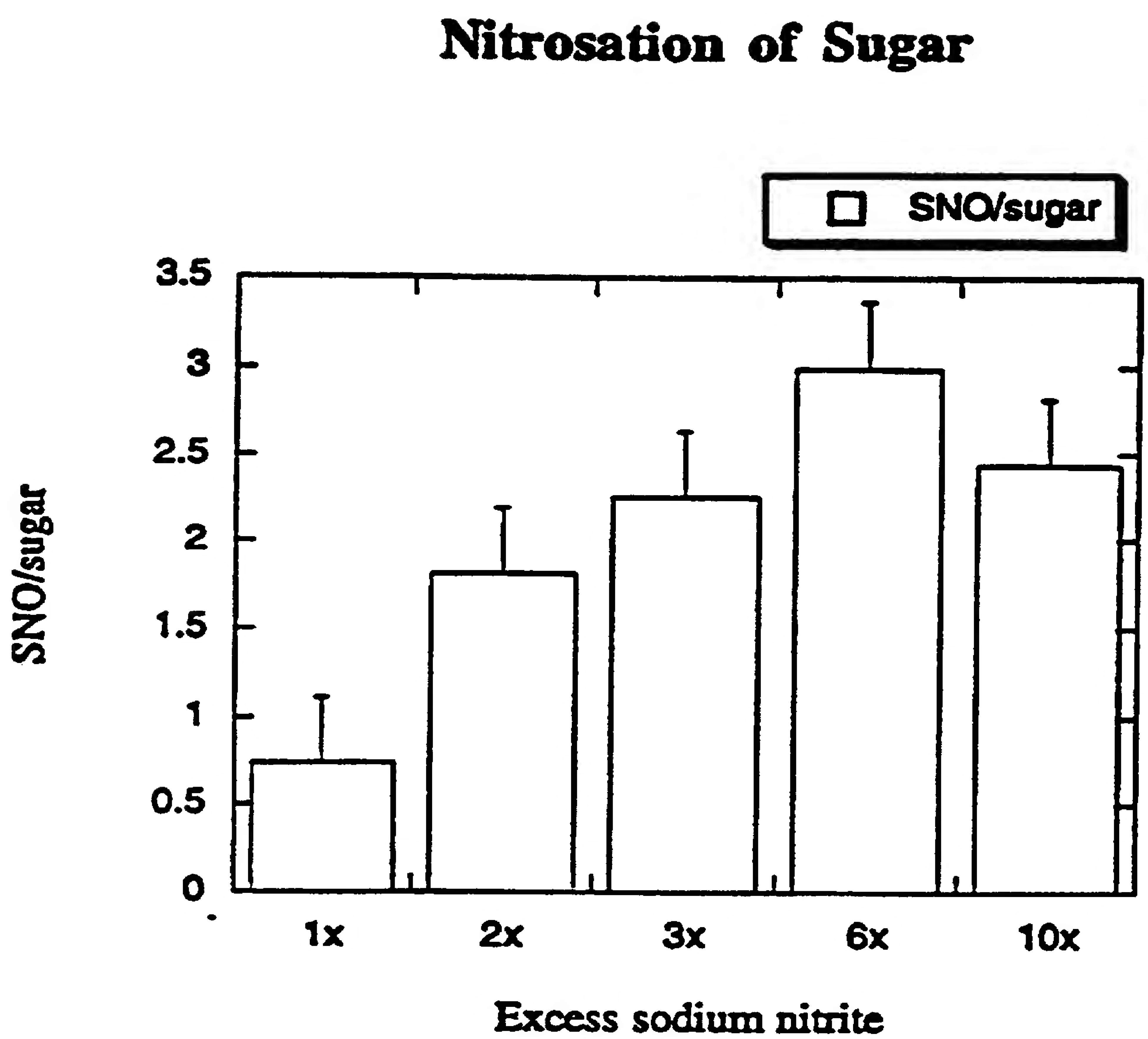

Nitrosation of Sugar
SNO/sugar
3.5
3
2.5
2
1.5
1
0.5
0
SNO/sugar
1x
2x
3x
6x
10x
Excess sodium nitrite

Figure 2

VISIBLE U/V SPECTRUM OF A REACTION MIXTURE COMPRISING β-CYCLODEXTRIN AND A 50 FOLD EXCESS OF ACID NITRITE

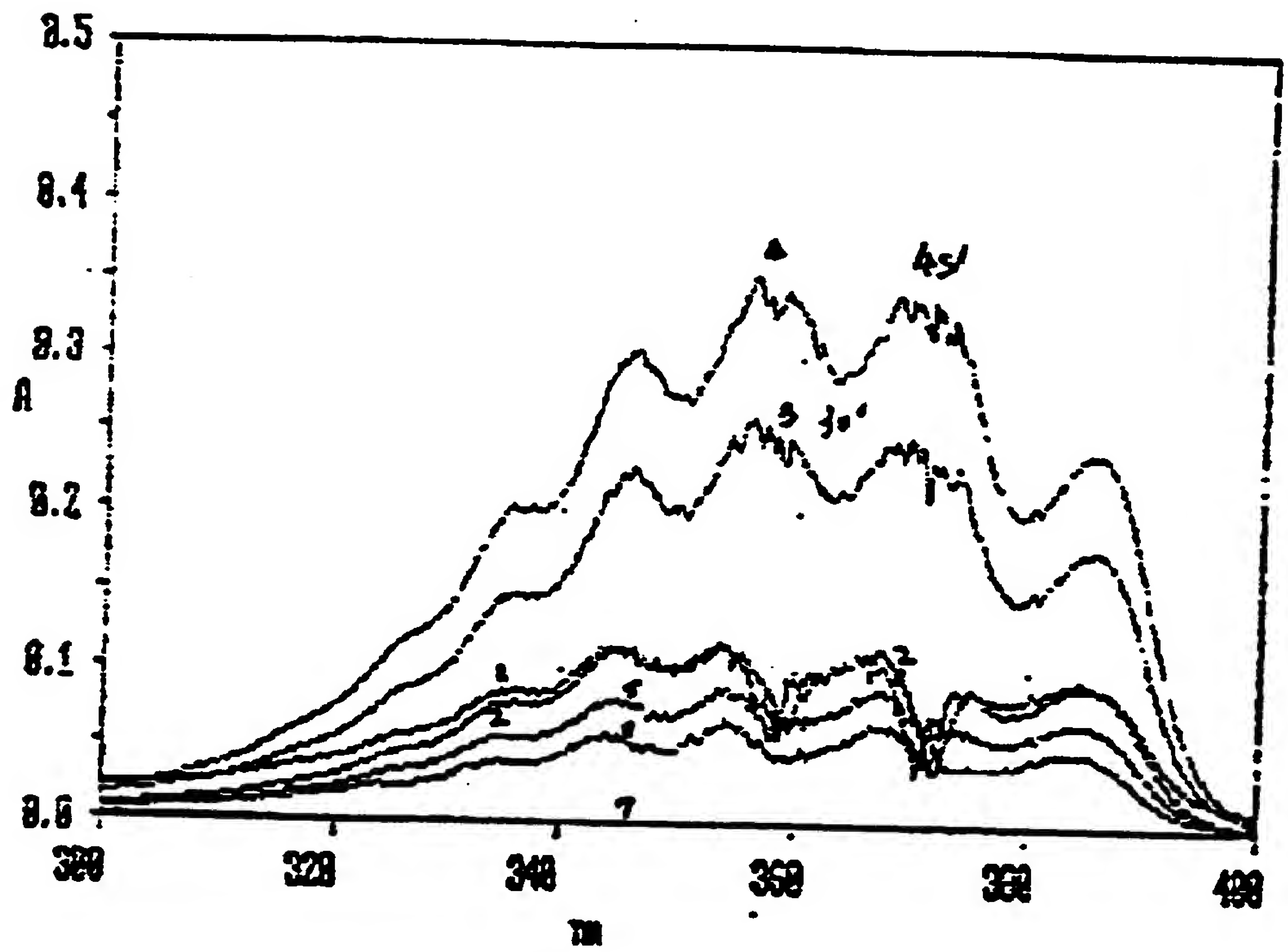


Figure 3

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 97/13475

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C08B37/00 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C08B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 114 506 A (CONSAGA ET AL.) 19 May 1992 see column 4, line 13 - line 23 see example I ---	1-6
X	US 4 138 535 A (SCHWEIGER R.G.) 6 February 1979 see column 5, line 9 - line 26 ---	1-4
Y	WO 96 02241 A (DUKE UNIVERSITY) 1 February 1996 see page 9, line 8 --- -/--	1-14, 17-28, 30-40, 51-54

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 1997	08.12.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lensen, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JONATHAN S. STAMLER ET AL.: "S-Nitrosylation of proteins with nitric oxide." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 89, 1 January 1992, WASHINGTON US, pages 444-448, XP000605487 see the whole document	1-14, 17-28, 30-40, 51-54
A	US 5 116 861 A (GOTO ET AL.) 26 May 1992	
A	US 5 405 919 A (KEEFER ET AL.) 11 April 1995	

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/13475

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 42-50, 55
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 42-50 and 55 are directed to a method of treatment of the human body by therapy (rule 39.1(4) PCT) the search has carried out and based upon the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/13475

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5114506 A	19-05-92	NONE	
US 4138535 A	06-02-79	US 4035569 A	12-07-77
		US 4143226 A	06-03-79
		US 4141746 A	27-02-79
		CA 979577 A	09-12-75
		DE 2120964 A	30-12-71
		US 3702843 A	14-11-72
WO 9602241 A	01-02-96	US 5583101 A	10-12-96
		AU 3008395 A	16-02-96
		CA 2194991 A	01-02-96
		US 5545614 A	13-08-96
US 5116861 A	26-05-92	CA 2022685 A	08-02-91
		DE 69008454 D	01-06-94
		DE 69008454 T	25-08-94
		EP 0412699 A	13-02-91
		JP 3261761 A	21-11-91
US 5405919 A	11-04-95	US 5525357 A	11-06-96
		US 5650447 A	22-07-97
		US 5632981 A	27-05-97
		US 5676963 A	14-10-97